AMP-activated protein kinase: structure and regulation.

Pascual Sanz

Instituto de Biomedicina de Valencia, CSIC, Jaime Roig 11, 46010-Valencia, Spain and Centre for Biomedical Research on Rare Diseases (CIBERER), Valencia, Spain.

*Corresponding autor:

Dr. Pascual Sanz
Instituto de Biomedicina de Valencia, CSIC
Jaime Roig, 11
46010-Valencia,
Spain
Tel. 3496-3391779
FAX. 3496-3690800
e-mail: sanz@ibv.csic.es

ABSTRACT

Mammalian AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status. It is activated by a large variety of cellular stresses that increase cellular AMP and decrease ATP levels and also by physiological stimuli, such as muscle contraction, or by hormones such as leptin and adiponectin. AMPK modulates multiple metabolic pathways. As a result, it has become a target for the development of new drugs for the treatment of type II diabetes, obesity or even cancer. In fact, it has been recently reported that drugs used in the treatment of diabetes, such as metformin and thiazolidinediones (TZDs), exert their beneficial effects through the activation of AMPK. AMPK is a heterotrimeric complex composed of a catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ). Functional orthologues of this kinase complex are found throughout eukaryotic kingdom, from yeast to humans, indicating that the function of this complex is evolutionarily conserved. This review summarizes the recent studies on the structure and regulation of the AMPK heterotrimeric complex.

Key words: AMP-activated protein kinase, Snf1, LKB1, CaMKK, phosphatase.

1.- INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status. It is activated in an ultrasensitive manner by cellular stresses that deplete ATP (concomitant increase in AMP levels), either by inhibiting ATP production (hypoxia, glucose deprivation, heat shock, mitochondrial oxidative phosphorylation inhibitors, etc.) or by accelerating ATP consumption (muscle contraction, etc.). Once activated, it switches on catabolic pathways and switches off many ATP-consuming processes (anabolic pathways) (see [1], [2], [3], [4], [5], for reviews). AMPK is a heterotrimer of three different subunits: α, β and γ. Phylogenetic analysis of the three subunits indicates that all of them have orthologues in the eukaryotic kingdom, from yeast to humans, thus indicating that the function of this complex is well conserved throughout evolution ([6], [7]). AMPK-α is the catalytic subunit of the AMPK complex; it contains a highly conserved kinase domain (KD), including the catalytic site, located at the N-terminus of the protein, and a regulatory domain (RD) located at the C-terminus of the subunit [6]. Two isoforms of the catalytic subunit have been described, namely AMPK-α1 and AMPK-α2; both are localized in the cytoplasm, although AMPK-α2 shows also a nuclear localization [8]. The AMPK-γ subunit contains four tandem repeats of a structure module called CBS, described initially in cystathionine-β-synthase [9], involved in AMP binding [10]. Three isoforms of the γ-subunit, named AMPK-γ1, γ2 and γ3, have been described; they differ at the N-terminal part of the proteins, whereas the C-terminus (where the CBS domains are located) is conserved in the three isoforms. AMPK-β subunit functions as a scaffold to assemble α and γ subunits, and also determines the subcellular localization and substrate specificity of the complex. Two isoforms of the β-subunit (AMPK-β1 and β2) have been described; they only differ at the N-terminus, but interact with the same efficiency with AMPK-α and AMPK-γ subunits ([11], [12]).

AMPK function has been implicated in multiple metabolic pathways. Several excellent reviews have recently appeared covering the involvement of AMPK in the function of different tissues, such as skeletal muscle [13], heart [14], liver [15], adipose tissue [16], hypothalamus [17], and in processes such as mitochondrial biogenesis [18] and cell proliferation [19]. To activate AMPK, two processes must occur: phosphorylation of the Thr172 residue in the catalytic domain and allosteric activation by AMP. The combination of these two effects causes a more than 1000 fold increase in

kinase activity [20]. In this review, I summarize the recent studies on the structure of the different AMPK subunits and also those involved in the regulation of the activity of the AMPK complex.

2.- STRUCTURAL STUDIES ON AMPK-α SUBUNIT

As mentioned above, two isoforms of the catalytic subunit of AMPK have been described, namely AMPK-α1 and AMPK-α2. AMPK-α1 is a protein of 548 aa (63 kDa) encoded by the PRKAA1 gene; AMPK-α2 is a protein of 552 aa (63 kDa) encoded by the PRKAA2 gene. Both subunits contain the kinase domain (KD) at the N-terminus (residues 1-312), plus a C-terminal regulatory domain (RD) (residues 313-548, in α1; 313-552, in α2) involved in the interaction with the AMPK-β and AMPK-γ subunits (see below). AMPK-α has a well-defined orthologue in the yeast *Saccharomyces cerevisiae* named Snf1. It is a protein of 633 aa (72 kDa) that also contains an N-terminal kinase domain (residues 1-391) and a C-terminal regulatory region (residues 392-633) (Fig. 1A).

The N-terminal kinase domain of the AMPK-α family is conserved throughout evolution; for example, it is 47% identical (61% homology) to that of the yeast Snf1 or plant SnRK1 (Snf1 related kinase) [6]. However, the C-terminal regulatory domain is divergent. The kinase domain contains the Thr172 residue whose phosphorylation is critical for activity (see below). It is assumed that in the non-phosphorylated inactive form of AMPK-α, the KD domain interacts with an auto-inhibitory region located in the RD domain (residues 313-392; AID) [6]. Interaction of AMPK-γ with the C-terminal domain of AMPK-α counteracts the function of the auto-inhibitory domain (AID) ([21], [22]) (Fig. 1A). Recently, the residues involved in auto-inhibition have been studied in more detail. It was found that a conserved short segment of the α-subunit (residues 313-335 in α1) was responsible for the auto-inhibition. In this sequence, several hydrophobic and charged residues, in particular Leu-328, were found to be critical for α1 auto-inhibition. It was also found that Leu328 interacted with Val298 in the kinase domain, and mutations such as L328Q or V298G that abolished the interaction, conferred a constitutive activity to a truncated from (residues 1-394) of the AMPK-α1 subunit. However, deletion of the (313-335) domain in the full length AMPK-α1 subunit did not fully activate the kinase domain. Therefore, additional interactions

between the kinase domain (1-312) and the regulatory domain (313-548) may also be responsible for the auto-inhibition [23].

Due to this auto-inhibitory reaction, short forms of the α subunits containing only the kinase domain (i.e. residues 1-312) have been constructed and used as constitutively active forms of the enzyme. Although these truncated forms are initially inactive, they become active upon phosphorylation of Thr172 residue or by replacing this residue by an Asp or Glu (T172D/E), mimicking the negative charge of the phosphate [24]. Alternatively, a recent report describes the isolation of small molecules that activate AMPK through antagonizing the auto-inhibition of α subunits. One of these small activators, named PT1, interacts with Glu96 and Cys156 residues, which are structurally close to the auto-inhibitory domain of AMPK-α1, and directly relieves auto-inhibition. PT1 enhances also AMPK phosphorylation at Thr172 without increasing the AMP/ATP ratio [25].

Several attempts were made initially to define the crystal structure of AMPK-α subunit. Two reports partially defined the crystal structure of the kinase domain of the yeast AMPK-α orthologue, Snf1. In the first report, the kinase domain of Snf1 (residues 41-315) (Snf1-KD) was crystallized and its structure determined by single-wavelength anomalous dispersion of selenomethionyl protein derivatives (PDB, 2EUE) (Fig. 2A). The structure shared strong similarity to other protein kinases (PKA, CaMK, etc), with a small N-terminal lobe (with five β-sheets and a α-helix) and a large C-terminal lobe mostly helical (6 α-helices). Two negative surface patches in the structure were defined as important for the recognition of the substrates by this kinase, and the regulatory Thr210 residue presumably located on the top of the cleft between the N-terminal and the C-terminal lobes (unfortunately, the structure is disordered in this area so the precise location of the Thr210 residue could not assigned) [26]. Other authors crystallized a similar kinase domain of Snf1 (residues 33-320) (PDB, 2FH9) (Fig. 2B). They found a similar bilobe fold and defined the ATP binding residues in a grove formed between the two lobes. However, and in contradiction with the previous crystal structure studies, these authors indicated that the kinase domain was able to form dimers, that they confirmed experimentally by gel-filtration and equilibrium centrifugation. The introduction of an I257E mutation, in the dimerization domain, prevented dimer formation. The authors also indicated that full-length Snf1 was able to form dimers and

suggested that the dimer conformation was inactive, since the catalytic site would be buried by the second protomer. It was proposed then that disruption of the dimer was necessary to gain full activation of the Snf1 kinase complex [27].

Recently the kinase domain of AMPK-α2 (residues 4-279) was crystallized (PDB, 2H6D) (Fig. 2C). Apparently, it displays a similar monomeric structure to Snf1 KD (residues 41-315) (unpublished results from the Structural Genomics Consortium).

3.- STRUCTURAL STUDIES ON AMPK-β SUBUNIT

Two isoforms of the AMPK-β regulatory subunit have been described, namely AMPK-β1 and AMPK-β2. AMPK-β1 is a protein of 270 aa (38 kDa) encoded by the PRKAB1 gene, and AMPK-β2 is a protein of 271 aa (34 kDa) encoded by the PRKAB2 gene (Fig. 1B)**.** They are 71% identical and differ only at the N-terminus [11]. AMPK-β subunits function as a scaffold to assemble α and γ subunits, *via* binding to their conserved KIS (kinase interacting sequence) and ASC (association with Snf1 complex) domains, respectively. The KIS and ASC domains are also present in yeast β-subunits [Gal 83 (Fig. 1B); Sip1 and Sip2], although these proteins show a divergent N-terminal domain. Recently, a new domain in the β-subunits has been defined: it is the glycogen binding domain, GBD, which extends from residues 68 to 163 inside the formerly defined KIS domain. GBD binds glycogen and can lead to abnormal glycogen-containing inclusions when the heterotrimeric complex is overexpressed. These inclusions are absent when GBD-truncated forms of β subunits or β subunits mutated in residues involved in glycogen binding (W100G, K126Q) are used ([28], [29]). The truncated β subunits lacking the entire GBD domain still form regular complexes with α and γ subunits, which are fully active and regulated normally, indicating that that GBD domain is not critical for activity. On the contrary, the ASC domain is essential for activity. Deletion of small parts of this domain leads to forms that can no longer assemble in complexes and are inactive, indicating that the ASC domain is required for the formation of a stable, active αβγ complex ([28], [29]).

AMPK-β subunits are phosphorylated at multiple sites, Ser24/25, Ser108 and Ser182 (AMPK-β2 lacks Ser24/25 site). Ser24/25 and Ser108 are autophosphorylated by AMPK-α1, whereas Ser182 is phosphorylated by an upstream AMPK kinase. Although none of the mutations in the phosphorylation sites of AMPK-β1 subunit affect the rate of phosphorylation of AMPK-α1 at Thr172, phosphorylation of Ser24/25 and

Ser182 residues is necessary for the nuclear exclusion of β1 subunit, and phosphorylation of Ser108, although it does not affect subcellular distribution of AMPK-β1, increases AMPK activity [30].

In addition, β-subunits are myristoylated at the N-terminus on Gly2 [31]. The elimination of the myristoylation site results in a more homogeneous distribution of the AMPK-β subunits inside the cell and in an increase in AMPK activity [30].

The crystal structure of AMPK-β1-GBD (residues 68-163) complexed with β-cyclodextrin has been determined (PDB, 1Z0M) (Fig. 3A). This GBD is remarkably similar to the major carbohydrate binding site of the starch binding domains in cyclodextrin glycosyltransferase, β-amylase and glucoamylase. This study defined the residues involved in the interaction with the carhohydrate, which were W100, K126, W133, L146 and N150. Five sugars can be accommodated in the structure of the GBD, suggesting that malto-pentaose is the smallest oligosaccharide that could prevent glycogen from binding to AMPK [32]. The Structural Genomics Consortium has also deposited the structure of AMPK-β2-GBD (residues 68-163) without β-cyclodextrin (PDB, 2F15). Apparently, this structure is similar to the one from β1-GBD.

4.- STRUCTURAL STUDIES ON AMPK-γ SUBUNIT

Three different AMPK-γ subunits have been described. AMPK-γ1 is a protein of 331 aa (37 kDa), AMPK-γ2 is a protein of 569 aa (63 kDa) and AMPK-γ3 is a protein of 492 aa (55 kDa), encoded by the PRKAG1, PRKAG2 and PRKAG3 genes, respectively. They differ at the N-terminal domain of the proteins, whereas the C-terminal is conserved in the three isoforms (Fig. 1C). They bind 8-azido-AMP, as an indication of AMP binding [33]. The γ-subunits contain four tandem repeats of a structure module called CBS (cystathionine-β-synthase) [9], involved in AMP binding. The yeast orthologue Snf4 (322 aa; 36 kDa) also contains four CBS domains in the structure (Fig. 1C). The importance of these regions is highlighted by the presence of disease-causing mutations in the CBS domains of several CBS-containing proteins including AMPK, cystathionine β-synthase, IMP dehidrogenase-1 and chloride channels CLC1 [10]. CBS domains occur in tandem pairs, refereed as Bateman domains (Fig. 1C), and build the corresponding allosteric nucleotide binding site (AMP, ATP or SAM, depending of the CBS-containing protein). Biochemical studies indicate that in AMPK-

γ, CBS1-CBS2 and CBS3-CBS4 each bind one AMP molecule with high affinity, while the binding of the second AMP molecule is cooperative. The AMP binding affinity is higher in complexes with γ1 and lower in complexes with γ3. CBS domains also bind ATP, but with lower affinity. However, higher levels of ATP can displace AMP from binding [10].

Recently, a conserved region of 20-25 amino acids immediately N-terminal to the first CBS domain (Pre-CBS) has been involved in the interaction with the β subunits and the formation of a functional active αβγ complex (Fig. 1C). In addition, sequential truncations from the C-terminus of the γ subunits indicate that deletion of any of the CBS domains prevents the formation of a functional complex with the α and β subunits [34].

γ-subunits are acetylated at the N-terminus. No other additional post-translational modification has been described to date [31].

Recently, the crystal structure of a CBS domain pair from human AMPK-γ1 (residues 182-325, comprising CBS3 plus CBS4) in complex with AMP and the pharmacological activator ZMP has been described (PDBs 2UV4 and 2UV5, respectively) [35]. The core of a CBS domain consists of a βαββα structure in which the three β-strands form a sheet with the two α-helices to one side (Fig. 3B). This structure is homologous to those of other CBS-domain pairs already described ([36], [37]). A nucleotide-binding site sits between adjacent CBS domains and is formed from strands β1 and β7 of CBS4 and β2 and β3 of CBS3. AMP binds in a pocket lined by the side chains of residues Thr200, Ile204, Val225 and Ile312, making hydrogen bonds with surrounding residues [35]. Similar results were derive from the crystal structure of the second Bateman domain of the yeast AMPK-γ orthologue, Snf4 (residues 179-322, CBS3 plus CBS4; PDB, 2NYC) [38].

5.- STRUCTURAL STUDIES ON THE HETEROTRIMERIC COMPLEX

In mammalian cells, co-expression of the three subunits is required for kinase activity. In the absence of one of the subunits, the other two are degraded, so extracts do not show significant levels of the expressed proteins. Therefore, the ternary complex stabilizes the kinase ([24], [39], [40]). Pull-down experiments have demonstrated the ternary nature of the complex: AMPK-α1 and AMPK-α2 associate with β and γ

subunits with similar intensity [39], and AMPK-β interacts with both AMPK-α and AMPK-γ subunits [41]. However, interaction between AMPK-α and AMPK-γ subunits is prevented in the absence of AMPK-β subunits ([42], [43]).

Truncation experiments of AMPK-α2 subunit indicate that the C-terminal 313-552 domain is involved in binding to β1/2 and γ1 ([12], [44]). Further studies shortened the sequence of AMPK-α1 responsible for binding to β1 to residues 313-473 [42] (Fig. 1A). In addition, it has been recently described that the N-terminal kinase domain of the α2-subunit also participates in the binding to the γ1-subunit [44].

Several dominant negative forms of AMPK-α have been constructed, i.e. K45R, D157N and T172A. In these cases, the substitution of critical residues located near or in the catalytic site completely abolishes the kinase activity of the enzyme (Fig. 2C). However, these forms are still able to bind to the AMPK-β and AMPK-γ subunits and their overexpression results in a titration of the β and γ subunits from endogenous complexes, leading to the inhibition of AMPK activity ([31], [40], [45]). Similar results were obtained with an N-terminal truncated form of AMPK-α1 containing residues 262-548 [39].

The AMPK-β subunit acts as a scaffold for α and γ binding [42]. In the AMPK-β1 subunit, the C-terminal 85 residues (residues 186-270; ASC domain, Fig. 1B) are sufficient to interact with the three γ and the two α subunits, and to form an active heterotrimer. The C-terminal 25 residues of β1 subunit (residues 246-270) still bind all three γ subunits but not the α subunits and deletion of only the C-terminal Ile270 precludes βγ association in the absence of α subunit [42]. In addition, a recent report indicates that the C-terminal region of β1 subunit (residues 186-270) can form a complex with γ1 subunit in the absence of detectable α subunits [46]. The fact that only the C-terminal part of the β-subunit is involved in binding to the other AMPK subunits was confirmed in plants, where it has been found that a new β-subunit isoform (AKINβ3), that lacks the N-terminal domain and also the GBD domain, is still able to interact with α and γ subunits and form an active hetrotrimeric complex [47]. These results also confirmed that the GBD domain is not required for activity (see above). Recently, and in contrast to previous reports, it was described that the β-subunits did not bind directly to the γ-subunits [44]; these authors suggested that the C-terminus of β2-subunit binds to the C-terminus of the α2-subunit, which in turn binds to the γ1-subunit,

but there is no direct interaction between the β2 and the γ1 subunits [44]. However, recent data on the crystal structure of the heterotrimeric complex (see below) does not support this later finding.

The use of AMPK complexes synthesized in bacteria has allowed the identification of different phosphorylation sites within α and β subunits. Using a complex containing an AMPK-α1 D157A mutation (that abolishes activity), it was found that an AMPKK preparation from liver phosphorylated the α1 subunit, but not the β1 or the γ1. However, when using wild type or α1T172E (constitutively active), phosphorylation at the β1 subunit, in addition to α1 subunit, was observed. This results indicated: i) α1 contains alternative residues to Thr172 that are phosphorylated by AMPKK, and ii) β1 phosphorylation is due mainly to autophosphorylation by the α1 subunit. By mass spectrometry analysis the residues that were phosphorylated by AMPKK in α1 were, Thr172, Thr258 and Ser485 (Ser491 in α2). Mutants were constructed altering these residues, but it was found that either T258D and S485D, or T285A and S485A in α1 did not affect overall AMPK activity [48]. However, recent results indicate that phosphorylation of Ser485α1/491α2 site by Akt/PKB or cAMP-PKA prevents LKB1 (a major AMPKK; see below) phosphorylation at SerT172, decreasing the activity of AMPK ([49], [50], [51]).

Mass spectrometry analysis also indicated that the residues phosphorylated in β1, were Ser96, Ser101 and Ser108 [48]. These results are in contrast to previous reports that indicated that Ser24/25, Ser108 and Ser182 were the major phosphorylation sites in β1 [30].

All the biochemical and genetic data on the architecture of the heterotrimeric complex have been confirmed by the release in the year 2007 of three reports on the crystal structure of the complex from both yeast and mammalian cells, although in all the cases the kinase domain and the auto-inhibitory region of the α subunit and the N-terminal domain of the β subunit are lacking (Table I). The first released structure corresponded to a complex composed by truncated forms of α and β subunits (residues α440-576 and β205-298) and the entire γ subunit (residues 1-334) from the yeast *Schizosaccharomyces pombe* (PDB, 2OOX) [52]. The second report corresponded to a complex composed by truncated forms of α and β subunits (residues α1 396-548 and β2

187-272) and the entire γ1 subunit (residues 1-331) from mammalian (rat) AMPK (PDB, 2V8Q) [53], and the third, corresponded to the heterotrimeric core of the yeast *Saccharomyces cerevisiae* SNF1 complex composed by a truncated form of α subunit (but with a larger C-terminal regulatory domain; residues 398-633 of Snf1), a truncated form of β subunit (but containing the GBD domain; residues 154-415 of Sip2) and the entire γ subunit (Snf4; residues 1-322), (PDB, 2QLV) [54]. The structures of the three core αβγ complexes are very similar (Fig. 4A). They are triangular with a base formed by the γ subunit which associates with a tight αβ complex (the C-terminal region of the α subunit forms a compact domain with the C-terminal region of the β subunit) that forms the apical domain. Structures show a direct βγ interaction mediated almost exclusively by hydrogen bonds and salt bridge interactions between three β-strands, two provided by the C-terminus of the β subunit and one corresponding to the N-terminal region of the γ subunit. The γ subunit forms an elliptical disk with an aqueous pore in the center. CBS domains 1 and 2 (first Bateman domain) form the interface with the αβ complex and are connected to CBS domains 3 and 4 (second Bateman domain) that lay underneath. However, there are also differences among the three structures. First, there is a significant difference in the positions of the α and β subunits relative to the γ subunit, corresponding to a rotation of about 12º in their relative orientations, of the yeast *S. pombe* structure in comparison to the mammalian and the yeast *S. cerevisiae* ones. Second, in the case of the *S. pombe and S. cerevisiae* structures, they contained dimers of trimers ([52], [54]), whereas the mammalian structure was monomeric [53].

As described above, pairs of CBS domains participate in the binding of AMP. Since CBS1 and 2 form the first Bateman domain and CBS3 and 4 form the second Bateman domain, it could be expected that a γ subunit could bind two molecules of AMP. However, since the two CBS motifs of the first Bateman domain also associate together in a pseudosymmetrical manner with the two CBS motifs of the second Bateman domain, two additional potential nucleotide-binding sites could be generated, making a total of four nucleotide-binding sites in each γ subunit. All four sites are located between pairs of adjacent helix-loop-strands with an Asp residue from the corresponding α-helix positioned to bind the nucleotide ribose hydroxyl residues, except for the site between CBS1 and CBS2, where an Arg is found instead of an Asp residue in the corresponding CBS2 chain. However, in the *S. pombe* structure only one adenine nucleotide is found, bound at the interface between CBS domains 3 and 4,

being the first Bateman domain (CBS1+2) unoccupied [52]. In the case of the *S. cerevisiae* structure, no nucleotide was initially found, although low-resolution data on a second crystal suggested the presence of one AMP in the same position (interface of CBS3+4) as in *S. pombe* [54]. In the case of the mammalian structure, three AMP are found [53]; one AMP is tightly bound and non-exchangeable in the interface of CB3+4, as in the yeast structures (AMP3 in Fig. 4B). However, two additional AMPs are located in the interfaces of CBS1+2 and CBS3+4 (AMP1 and AMP2 in Fig. 4B). These AMPs are interchangeable by ATP and are responsible for the adenyl-sensing properties of the mammalian enzyme. One site formed by the interface of CBS1+2 is unoccupied in the mammalian structure (AMP4 in Fig. 4B), maybe because of the absence of a conserved Asp residue in CBS2 positioned to bind the nucleotide ribose ring. Since the yeast structures do not contain AMP in sites corresponding to AMP1 and AMP2 (Fig. 4B), this could be the reason why these enzymes do not respond to AMP. Recently, a new structure of *S. pombe* AMPK heterotrimer complexed with ADP has been described. In this case, two ADPs are found, one occupying the original AMP site (AMP3 in Fig. 4B) and the other in the unoccupied interface of CBS1+2 (AMP4 in Fig. 4B) [55]. The significance of this finding is unclear, since no biochemical studies have been done on the *S. pombe* AMPK complex and no information is available on whether ADP could affect enzyme activity.

Since the structure of the *S. cerevisiae* SNF1 complex contains larger fragments of the α (Snf1) and β (Sip2) subunits, it defines new structural features not present in the other structures. First, the GBD domain of the β subunit (residues 154-247 of Sip2) has close interactions with the rest of the heterotrimer, primarily with the N-terminal region and an α-helix of CBS2 of Snf4 (Fig. 4A). The putative binding site for carbohydrates is exposed to the solvent and should be available for binding ligands in this complex. This feature is interesting although, in the case of the mammalian β subunit, the GBD plays no role in the regulation of AMPK activity (see above). Second, residues 460-495 of Snf1 (α subunit), corresponding to the regulatory region (RD), have well defined interactions with Snf4 (γ subunit) (residues 467-469 of Snf1 interact with residues 270-275 of the first β-strand of CBS4 of Snf4) (Fig. 4A). This demonstrates a direct interaction between the α and the γ subunits in SNF1 complex, which is fully supported by earlier biochemical and genetic data [54]. The authors suggested that the structure having the RD sequence bound to γ subunit should correspond to the active

conformation of the SNF1 complex. It remains to be determined how the binding of different ligands to the γ subunit can control its interaction with the RD sequence and how this RD sequence is recognized by the protein kinase domain in the inactive conformation of the complex. In spite of this, the interaction between the RD sequence and the γ subunit is particularly interesting since a disease-causing mutation in the mammalian γ2 subunit (see below), namely N488I (equivalent to Asn251 on Snf4), maps in the same region and could be affecting the binding of the γ2 subunit to the C-terminal regulatory domain of α subunit, explaining in this way the absence of regulation of the mutated γ2 subunit [54].

Mutations in the γ subunit have been associated with disease. Up to ten different point mutations in the mammalian γ2 subunit cause heart disease characterized by excessive glycogen storage in cardiac myocytes, what leads to ventricular pre-excitation [56]. All these mutations reduce binding and activation by AMP [10]. Since the structure of the γ subunit is now available, it has been possible to map the position of the residues involved in pathogenesis. In this way, it has become clear that nine out of the ten mutations that have been characterized involve amino acids lining the interior surface of an internal cavity formed in each Bateman domain and, in the case of γ2Arg302, γ2His383, γ2Arg384, γ2Thr400, γ2Arg531, their side chains are in the proximity to the adenyl-binding sites.

When comparing the structures of the heterotrimers obtained in the presence of AMP or ATP, no major difference is observed in terms of movement of the corresponding CBS domains. This was quite unexpected since it was anticipated to see a change in the conformation of the CBS domains depending on which nucleotide was bound. Perhaps the absence of critical domains in the crystallized core structures, such as the catalytic and autoinhibitory regions of α subunit, could account for this unexpected result. This leaves open the question of how the binding of AMP activates the AMPK complex. However, very recently, an answer to this question has been provided [57]. These authors purified large quantities of mammalian heterotrimeric complexes containing, this time, full length untagged α, β and γ subunits from mammalian AMPK and described the biophysical characterization of the heterotrimers by small angle X-ray scattering in solution, transmission and scanning transmission electron microscopy and mass spectrometry. All these techniques revealed that mammalian AMPK heterotrimers were elongated flat particles with protrusions and

indentations. More importantly, addition of AMP resulted in a significant change in the conformation of the heterotrimer towards a more compact structure. The change in the conformation of the γ subunit upon AMP binding could be transmitted via the regulatory domain (RD) of the α subunit onto the kinase domain: the RD could be relocated, releasing in this way the kinase domain from its auto-inhibition. These changes may also alter the accessibility of the phospho-Thr172 residue, thus positively affecting AMPK activity [57].

6.- RELATIONSHIP BETWEEN MAMMALIAN AMPK AND YEAST SNF1 COMPLEXES.

Due to the considerable similarity between the mammalian AMPK and the yeast SNF1 complex, the yeast system has been used to investigate AMPK regulation. Attempts have been made to complement SNF1 subunit deficiencies with their mammalian counterparts, however full recovery of the corresponding *snf1* phenotype has never been achieved. In this way, expression of AMPK-α1 or α2 in yeast does not complement *snf1* mutants [41], expression of AMPK-γ1, γ2 or γ3 does not complement *snf4* mutants [41] and expression of AMPK-β1/2 does not complement *sip1 sip2 gal83* mutants (personal observation). These results were quite surprising since AMPK-β interacts with Snf1 and Snf4, AMPK-γ interacts with Snf1 and Gal83 and AMPK-α interacts with Gal83 although it does not interact with Snf4 (personal observation).

Chimeras containing the N-terminus of AMPK-α1 (residues 2-261) and the C-terminus of Snf1 (residues 300-633) (α1-Snf1), and also the opposite, N-terminus from Snf1 (residues 2-299) and C-terminus from AMPK-α1 (residues 262-548) (Snf1-α1) have been constructed. Two-hybrid analysis revealed that the α1-Snf1 chimera associated with Sip2 and Snf4, but not with the mammalian counterparts, and that the Snf1-α1 chimera associated with mammalian β1 and γ1 subunits, but not with the yeast counterparts [58]. This indicates that the C-terminal part of the α subunit defines a cell-specific interaction profile with the corresponding β and γ subunits. This is in agreement with the fact that α-subunits are more divergent at their C-terminus (see above). The chimeras were expressed in mammalian cells and it was found that, as in the case of regular AMPK-α1, the expression of Snf1-α1 chimera was dependent on the co-expression of AMPK-β1 and AMPK-γ1 subunits, whereas the expression of α1-Snf1 chimera in mammalian cells was dependent on the co-expression of yeast Sip2 and

Snf4. The Snf1-α1 chimera produced inactive heterotrimeric complexes. However, the α1-Snf1 chimera showed significant kinase activity that was unaffected by AMP. Since the SNF1 complex is not allosterically activated by AMP [59], it was concluded that the C-terminal domain of AMPK-α1 (absent in the chimera) was involved in AMP regulation [58]. This α1-Snf1 chimera was able to form a functional kinase complex when expressed in yeast, via association with the endogenous regulatory subunits, and was also able to display significant kinase activity under low-glucose conditions [58].

7.- REGULATION OF AMPK ACTIVITY

7.1.- AMPK activation.

The AMP-activated protein kinase is a sensor of cellular energy charge. This system is activated in an ultrasensitive manner by cellular stresses that increase the AMP/ATP ratio. AMP activates mammalian AMPK by two mechanisms: i) allosteric activation of AMPK, and ii) binding to AMPK making it a worse substrate for phosphatases. This allows a >200 fold activation of AMPK. All these effects are reversed by ATP, indicating that AMPK has a single allosteric site that binds both AMP and ATP in a mutually exclusive manner ([6], [20], [60], [61], [62]).

Activation of AMPK correlates with phosphorylation at the Thr172 within the "activation loop" of the kinase domain ([63], [64]). This phosphorylation is achieved by an upstream AMPK kinase (AMPKK; see below). Phosphorylation of Thr172 is necessary and sufficient for AMPK activity, since a T172A mutant shows no enzymatic activity, whereas a T172D mutant possesses detectable activity (40-50% of regular activity) which is not affected by phosphatase treatment [65]. The modification of the Thr172 does not affect interaction with the β and γ subunits. For this reason the T172A mutant can be used as a dominant negative form of the AMPK-α subunit [31]. These results are in contrast with the ones obtained in the yeast orthologue Snf1 (α-subunit), where it has been found that, although a change in the corresponding Thr172 (T210A) eliminates SNF1 activity, the mutated form is unable to interact with Snf4 (γ-subunit) [21].

In yeast, regulation of the activity of the Snf1 complex involves two steps, one that requires phosphorylation of the Snf1 catalytic subunit (α-subunit) by an upstream protein kinase and another that is mediated by the interaction between Snf4 (γ-subunit) and Snf1 (α-subunit) [21]. Phosphorylation of Thr210 can occur independently of the

Snf4 step, but to achieve full activity, the complex has to undergo a Snf4-dependent rearrangement [66]. Interaction with Snf4 is required for efficient activation because it counteracts the function of an auto-inhibitory domain present in the C-terminus of the α-subunit ([21], [22]). After these two steps, the phosphorylated Snf1 kinase (Thr210-P) opens its active site, making it accessible to substrates (Fig. 5A). Regulation of AMPK activity follows a similar trend, including phosphorylation of a conserved Thr172 residue of AMPK-α by an upstream kinase and AMPK-γ dependent allosteric regulation by AMP. The identification of the putative AMPK upstream protein kinase was a frustrating project until the discovery of yeast Snf1 upstream kinases: Sak1, Elm1 and Tos3 ([67], [68], [69]). They are monomeric protein kinases, being Sak1 the most important upstream kinase in the glucose repression pathway and the only one that forms a stable complex with Snf1 ([70], [71], [72]). Sak1, Elm1 and Tos3 are members of the ELM family of protein kinases [73]. While none of them had a clear mammalian homologue, their kinase domains were most closely related to those of the protein kinase LKB1, and the calmodulin-dependent protein kinase kinases CaMKKα and CaMKKβ. Evidence came rapidly indicating that all three, but especially LKB1 and CaMKKβ, phosphorylated AMPK-α at Thr172 and thus activated AMPK *in vivo* ([74], [75], [76], [77]). The relationship between the upstream kinases in yeast and mammals was reinforced when it was described that the expression in yeast of mammalian LKB1 and CaMKKα phosphorylated and activated Snf1 at its corresponding Thr210 site [78]. Recently, a genetic selection for mammalian Snf1-activating kinases in yeast identified TAK1 (transforming growth factor-β-activated kinase), a member of the mitogen-activated protein kinase kinase kinase family (MAPKKK), as an additional AMPK upstream kinase [79] (Fig. 5B).

7.1.1.- AMPKK: LKB1

LKB1 is a tumor suppressor kinase related to the Peutz-Jeghers cancer syndrome [80]. As mentioned above, it is an upstream AMPK kinase (AMPKK), since it can phosphorylate *in vitro* AMPK [68], and it accounts for most of the AMPKK activity detectable in cell extracts [1]. LKB1 is found in complexes with STRADα/β (Ste20 Related Adaptor; an inactive pseudokinase) [81] and MO25α/β (mouse protein 25; an armadillo-repeat scaffolding-like protein) [82]. The ternary complex is required for activity since in the absence of STRAD or MO25, no activity is observed. The crystal

structure of MO25α in complex with the C-terminus of STRADα has been recently described and indicates that MO25α acts as a scaffold protein for binding of STRAD/LKB1, LKB1 substrates or other regulatory components of the system [83]. Structurally, the kinase domain of LKB1 binds to the pseudokinase domain of STRAD, and MO25 binds to the C-terminal residues of STRAD, stabilizing the binding of STRAD to LKB1 ([81], [82]). Mutations in LKB1 isolated in Peutz-Jeghers patients fail to interact with STRAD-MO25, thus defining one of the reasons for this pathology [84]. LKB1 is truly an AMPK kinase since immunoprecipitation with anti-LKB1 antibodies reduces AMPK activity and recombinant complexes of LKB1-STRAD-MO25 activate AMPK via phosphorylation of AMPK-α at Thr172 in the activation loop. It has also been found that chemical activators of AMPK activity such as AICAR and phenformin (see below) do not activate AMPK in cells lacking LKB1 (i.e. HeLa cells), but activation can be restored by stably expressing LKB1 in these cells. The same results were obtained with fibroblasts from LKB1 knock-out mouse embryos: neither AMP nor phenformin directly stimulated the AMPK complex. All these results suggest that LKB1-STRAD-MO25 is a major upstream regulator of AMPK ([74], [75], [85], [86]).

LKB1 is the major upstream activating kinase for AMPK in skeletal muscle. Using mice in which expression of LKB1 has been reduced to only 10% in skeletal muscle or completely abolished (LKB1 skeletal muscle conditional knock-out), it was observed that LKB1 was required for AICAR-induced activation of AMPKα2. LKB1 was also required for contraction-induced AMPK activation and also for contraction-induced glucose transport in skeletal muscle. These results indicate that contraction-induced glucose uptake operates via an LKB1 dependent pathway, that could activate AMPK or some other AMPK related kinases to improve glucose uptake [87].

LKB1 is also the major upstream activating kinase for AMPK in liver. Liver specific deletion of LKB1 reduces substantially AMPK phosphorylation and activity, which leads to a marked hyperglycemia due to an increased hepatic gluconeogenic and lipogenic gene expression. However, peripheral glucose uptake (skeletal muscle) was not affected. LKB1 deletion increased expression of gluconeogenic genes (such as PEPCK and G6Pase) due to an increase in expression of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), whose expression was improved by a better activity of the CREB transcription factor. It has been recently described that the activity of CREB is regulated positively by TORC2 (transducer of regulated CREB

activity 2) and that AMPK and other related kinases phosphorylate TORC2 promoting its inactivation and sequestration by 14-3-3 proteins in the cytoplasm. As a result of LKB1 deficiency, lower AMPK activity leads to more unphosphorylated TORC2 that may activate CREB, which in turn activates PGC-1α, thus activating the expression of gluconeogenic genes. Lipogenesis was also improved in LKB1 knock-out animals, since lower AMPK activity leads to the improved expression of sterol regulatory element binding protein 1 (SREBP1), which improved the expression of target genes (FAS, ACC1, L-PK) [88], as AMPK is a negative regulator of SREBP1 [89].

7.1.2.- Alternatives to LKB1: CaMKK

LKB1 is related to activation of AMPK caused by a change in the AMP/ATP ratio. However, there are examples were AMPK is activated without changes in this ratio: a) During mild ischemia the ratios AMP:ATP and creatin/creatin-P are not altered, but AMPK activity and AMPKK activities are increased [90]; b) In several cell lines that do not express LKB1 (i.e. HeLa cells), AMPK is activated and phosphorylated in response to growth factors, such as Insulin growth factor 1 (IGF-1), by a LKB1-independent pathway [91].

The characterization of additional upstream kinases for activating AMPK came from the observation that the AMPKK activity present in LKB1-deficient cells was stimulated by Ca^{++} ionophores and inhibited by STO-609, a specific inhibitor of CaMKK, and by specific siRNAs corresponding to CaMKKβ. It was then defined that CaMKKβ was an additional upstream AMPK kinase, that also phosphorylated AMPK-α at residue Thr172. It is worth pointing out that CaMKKs are significantly expressed in neurons, where activation of AMPK may be a mechanism for anticipating the increased demand for ATP that always accompanies rises in cytosolic Ca^{++} [4]. In fact, K^{+} induced depolarization in rat cerebrocortical slices, which increases intracellular Ca^{++} without disturbing cellular adenine nucleotide levels, activates AMPK and this is prevented by STO-609. Therefore, there is a potential Ca^{++}-dependent neuroprotective pathway involving phosphorylation and activation of AMPK by CaMKKβ ([76], [77], [92]).

These results indicate the existence of two converging pathways of AMPK regulation: one directed by LKB1, which is affected by changes in AMP levels, and another directed by CaMKKs, dependent on changes in intracellular Ca^{++}. Since AMPK

(AMP-activated protein kinase) is additionally activated by Ca^{++}, some authors have suggested a new name for this protein: PKE, for protein kinase energy or economy kinase [93].

7.1.3.- Alternatives to LKB1: TAK1.

As described above, a genetic selection for mammalian Snf1-activating kinases in yeast identified TAK1 (transforming growth factor-β-activated kinase) as an additional AMPK upstream kinase [79]. TAK1 is able to phosphorylate AMPK-α at Thr172, and expression of TAK1 and its binding partner TAB1 in HeLa cells (that lack LKB1) or treatment of these cells with cytokines (which stimulates the TAK1 signalling pathway) stimulated phosphorylation of Thr172 in AMPK-α [79]. However, more work needs to be done to determine the importance of this upstream kinase in cellular physiology.

7.2.- Methods for activating AMPK.

In yeast, the activity of the Snf1 kinase is regulated by glucose: in low glucose growing cells, the Snf1 kinase is active (phosphorylated), whereas in high glucose containing media, the Snf1 kinase is inactive (dephosphorylated) [21]. Signalling requires uptake and phosphorylation of the sugar, but unlike mammalian AMPK, Snf1 is not activated directly by AMP [59]. Similarly, mammalian AMPK can be activated by subjecting the cells to different stress conditions or by treating them with different activating compounds. The activating conditions of AMPK can be classified in two groups, those that increase the AMP/ATP ratio, and hence activate AMPK via LKB1, and those where the activation of AMPK is independent of the AMP/ATP ratio, where alternative AMPKK are involved [62] (Table II):

7.2.1.- AMPK activating compounds that increase AMP/ATP ratio.

AICA-riboside, 5-aminoimidazole-4-carboxamide-riboside (0.5-2.5mM). It is an activator of AMPK because it mimics the effects of AMP. It is cell permeable and produces ZMP after phosphorylation, an AMP analogue and an activator of AMPK. Activation occurs after 30 to 60 min and correlates with phosphorylation of Thr172 in AMPK-α [94]. Although an important tool, AICAR's utility is limited because: i) a triply phosphorylated form (ZTP) may also accumulate and act as an ATP analogue,

thus inhibiting AMPK in the long term; ii) ZMP mimics the effects of AMP on other AMP-sensitive enzymes, such as glycogen phosphorylase and fructose-1,6-bisphosphatase; iii) AICAR may also act via adenosine receptors, adenosine transporters or both [95]. In this way, AICAR can lead to the AMPK-independent activation of glycogen phosphorylase, by an allosteric mechanism not shared by metformin [96]. AICAR can also compete with adenosine for uptake by the nucleoside transporter, resulting in an increase in extracellular adenosine and subsequent activation of adenosine receptors, by AMPK-independent mechanisms [97]. Additional AMPK-independent effects of AICAR have been described recently ([98], [99], [100], [101], [102]). Therefore, when using AICAR, alternative activating AMPK conditions should always be used to make sure that the observed effect is dependent on AMPK activity.

Dinitrophenol (0.5mM) and **potassium cyanide** are mitochondrial inhibitors that activate AMPK by a mechanism involving an increase in AMP:ATP ratio and phosphorylation of AMPK-α at T172 ([103], [104]).

Oxidative stress (0.6-1mM H_2O_2) activates AMPK by a mechanism involving an increase in AMP:ATP ratio. Activation correlates with phosphorylation of AMPK-α at Thr172 ([58], [105]).

Inhibitors of the MAP kinase (ERK) cascade PD98059 and U0126 activate AMPK by increasing the AMP:ATP ratio [106].

Thiazolidinediones (TZDs) are activators of the peroxisome proliferator-activated receptor γ (PPARγ) in adipose tissue. TZDs are inhibitors of respiratory chain complex I; TZDs reduced cell respiration and increased anaerobic respiration [107]. Rosiglitazone, (200μM), a thiazolidinedione used in the treatment of type 2 diabetes, activates AMPK (both α1 and α2) in muscle by a mechanism involving an increase in the AMP:ATP ratio and phosphorylation of AMPK-α at Thr172 [103]. Pioglitazone and troglitazone have the same effect ([108], [109]).

Leptin, a hormone secreted by adipocytes, regulates food intake, energy expenditure and has neuroendocrine function. It stimulates fatty acid oxidation and glucose uptake in liver, pancreas and skeletal muscle and prevents the accumulation of lipids in non-adipose tissues. Leptin stimulates AMPK activity in skeletal muscle. Activation of AMPK correlates with an increase in the phosphorylation of Thr172 of AMPK-α2. Leptin activates AMPK in a biphasic fashion, through AMP:ATP dependent and independent mechanisms [110]. Mice deficient in leptin or leptin-receptors contain

lower AMPK activity in muscle and/or liver [111]. However, leptin inhibits AMPK in the hypothalamus [112].

Adiponectin (ACRP30), another hormone secreted by adipocytes, regulates energy homeostasis and glucose and lipid metabolism (stimulates β-oxidation and glucose uptake). It increases the AMP:ATP ratio, leading to phosphorylation at Thr172 an activation of AMPK [113]. Adiponectin also inhibits hypertrophic signaling in the myocardium through activation of AMPK signaling ([112], [114]).

α-Lipoic acid. Similar to leptin, α-lipoic acid has two separate functions. On one hand, it is able to activate AMPK in skeletal muscle, improving fatty acid oxidation and insulin-stimulated glucose uptake [115]. On the other hand, α-lipoic acid is able to inhibit AMPK activity at the neuronal level ([112], [116]).

7.2.2.- AMPK activating compounds independent of AMP/ATP ratio.

Peroxynitrite (ONOO-) increases AMPK activity (increase in Thr172 phosphorylation) without changing cellular AMP. AMPK activation involves the c-Src/PI3-kinase pathway ([117], [118]).

Metformin, (2 mM), a drug currently used in treatment of type 2 diabetes [119], activates AMPK *in vivo*. Activation occurs after 30 to 60 min and causes a Thr172 phosphorylation in both AMPK-α1 and α2. Activation of AMPK by metformin is mediated by mitochondrial reactive nitrogen species (RNS); depletion of mitochondria or overexpression of superoxide dismutases, as well as inhibition of endothelial nitric-oxide synthase, abolished metformin-enhanced phosphorylation of AMPK. In eNOS knock-out mice, metformin is unable to activate AMPK. These data suggest that RNS generated in mitochondria by metformin is required for AMPK activation *in vivo*. This activation requires the c-Scr/PI3Kinase pathway [120]. Phenformin (2 mM) has a similar effect as metformin, but actives AMPK more rapidly [74]. As mentioned above, LKB1 is also required for the activation of AMPK by metformin [88] and phenformin [87] in different tissues.

Osmotic stress (0.4-0.6M sorbitol) also activates AMPK (with Thr172 phosphorylation) without changing the AMP:ATP ratio; it acts via CaMKK to activate AMPK ([58], [103], [121]).

Estradiol. AMPK activation by estradiol was independent of both AMP levels and ERα (estrogen receptor α), but required estradiol conversion to its catechol metabolites [122].

Low levels of glucose. Similarly to the yeast SNF1 complex, low glucose improves AMPK activity. The activation correlates with an increase in the phosphorylation of Thr172 and does not depend on the levels of AMP or ATP [123].

Long-chain saturated and monounsaturated fatty acids (palmitate C16, stearate C18, oleate C18:1) (0.5mM) activate AMPK in heart inducing phosphorylation of Thr172 by a mechanism not dependent on the AMP:ATP ratio. Activation of AMPK by palmitate [124] was prevented by treating the samples with PP2C phosphatase, leading to the conclusion that exposure to fatty acid caused either an activation of an AMPK upstream kinase or an inhibition of an AMPK phosphatase [125].

The **release of nucleotides** (ATP, ADP and UTP, but not UDP) from damaged tissues induces phosphorylation and activation of AMPK in HUVEC cells, by a mechanism not linked to changes in AMP:ATP ratio. AMPK activation is mediated by P2Y1, P2Y2 and P2Y4 nucleotide receptors. Adenosine also induces phosphorylation of AMPK that was not mediated by P1 receptors, but required adenosine uptake [126]. Adenosine uptake is mediated by CNT2 transporters; therefore, nucleoside transporters might be novel players in the complex regulation of AMPK and energy metabolism [127].

AMPK is also activated by **agonists of different membrane receptors**. Stimulation of Gq-coupled receptors (GCRP) specifically activates AMPK-α1. In this sense, platelet-activation factor, norepinephrine and bradykinin activate AMPK when they bind to their respective Gp-coupled receptors [128]. Activation of AMPK in skeletal muscle is dependent on α1-adrenoceptors and not on α2- or β-adrenoceptors, being the AMP:ATP ratio unaltered after α1-adrenergic stimulation [129]. On the other hand, in 3T3-L1 adipocytes, activators of adenylate cyclase (forskolin or β-adrenergic agonist isoproterenol) or analogues of cAMP promoted phosphorylation and activation of AMPK, which was prevented by insulin [130]. Activation of AMPK in brown adipocytes is mediated by β- but not α-adrenoceptors and is independent of UCP1 [131].

Recently, a new activator of AMPK, namely **A769622,** has been described [132]. A769622 activates AMPK both allosterically and by inhibiting dephosphorylation of AMPK on Thr-172. Its mechanism of action does not involve binding to the γ subunit, as AMP does. However, its allosteric effect is abolished in an AMPK complex lacking the glycogen binding domain of the β subunit, and a mutation of Ser108 to alanine in this β subunit almost completely abolishes activation of AMPK by A769662 ([133], [134]).

In addition, it has been recently described that natural plant products with beneficial metabolic effects in diabetic and insulin-resistant states, such us **berberine** [135], **resveratrol** (present in grapes and red wine) [136], **epigallocatechin-3-gallate** (present in green tea) [137], **theaflavins** (present in black tea) [138], **caffeic acid phenethyl ester** (present in honeybee propolis) [139], **cucurbitane triterpenoids** (present in bitter melon) [140], activate AMPK. However, in most cases the mechanism of activation is unclear.

7.3.- AMPK inactivation.

In yeast, the activity of the three upstream kinases (Sak1, Elm1 and Tos3) seems to be constitutive, so the regulation of the phosphorylation status of Snf1 depends on the phosphatase involved in the dephosphorylation of Thr210. In this sense, some reports indicate that the yeast PP1 protein phosphatase, Glc7, and its regulatory subunit, Reg1, are responsible for the dephosphorylation and inactivation of the Snf1 protein kinase, in response to glucose ([141], [142]). In the absence of Reg1, Glc7 can not perform its function, so the Snf1 kinase complex is constitutively in the active, phosphorylated state, even in the presence of glucose ([141], [142]) (Fig. 5A).

As described above, activation of the AMPK complex requires phosphorylation of AMPK-α subunit at Thr172 by an upstream kinase. Studies on how these upstream kinases are regulated indicate that they are constitutively expressed and that increases in AMP do not stimulate their activity; only a rise in Ca^{++} activates CaMKK's ([75], [85]), thus leaving open the question of how phosphorylation of AMPK-α is regulated. Perhaps, and as in the case of the yeast Snf1 complex, the regulation depends on the activity of the phosphatase involved in the dephosphorylation of Thr172 (Fig. 5B). Despite several attempts made to characterize this phosphatase, its identity is still

unknown. *In vitro* studies have identified PP1, PP2A and PP2C protein phosphatases as possible candidates ([48], [143]). In addition, it has also been described that PP2A is involved in the regulation of the interaction of AMPK-α and AMPK-γ by glucose [12]. Also, treatment of primary hepatocytes with different protein phosphatase inhibitors (okadaic acid (OA) 100 nM, microcystine 300 nM, calyculin A 100 nM, cantharidin 30 μM, tautomycin 1 μM) produces the same high molecular weight AMPK-α forms compatible with phosphorylated proteins, also present in AICAR treated cells. The appearance of these forms correlates with higher AMPK activity measured as phosphorylated ACC (Ser79). The presence of Naringin (100-300 μM), a flavonoid antagonist of OA action, prevents the phosphorylation of AMPKα induced by OA, microcystin and AICAR, but not that produced by calyculin A or cantharidine, suggesting that AMPK-α may be activated by two independent mechanisms, one being naringin-sensitive (PP2A-associated) and the other being naringin-resistant (PP1-associated). Toxins (and AICAR) do not stimulate AMPK phosphorylation by LKB1 activation, but rather by increasing the susceptibility of AMPK to be phosphorylated by LKB1. Naringin could act on AMPK itself (acting as an AMP antagonist) or on a protein kinase involved in permissive AMPK phosphorylation [144]. Additionally, it has been recently reported that activation of protein phosphatase 2A by palmitate inhibits AMPK, suggesting a negative role of PP2A in the regulation of AMPK activity in vivo [145].

Recently it has been suggested that AMP, in addition to allosterically stimulating AMPK activity, could inhibit all phosphatases acting on AMPK, since it can prevent the *in vitro* dephosphorylation of Thr172-P in AMPK-α by PP2Cα. This effect requires a functional AMPK-γ subunit, since AMP can not prevent the dephosphorylation of Thr172 in αβγ complexes containing forms of the γ subunit that bind AMP poorly. According to this model, increased Thr172 phosphorylation by LKB1 would occur in response to decreased dephosphorylation following a rise in AMP. Dephosphorylation and inactivation of AMPK would occur when the concentration of AMP returned to basal levels [62].

7.4.- Methods for inhibiting AMPK activity.

There are several methods for inhibiting AMPK activity (TableIII):

AMPK-α1 K45R, D157A and T172A mutant forms may be used as dominant negative alleles of AMPK since they compete with endogenous AMPK-α for the binding of β and γ-subunits ([40], [45], [146]).

Creatine phosphate and ATP inhibit AMPK. This effect is entirely allosteric; they do not affect AMPKK ([60], [64]).

Compound C is a reversible inhibitor of AMPK [89], although it inhibits additional protein kinases and has AMPK-independent effects [147]. Caution has to be taken when using this compound because it inhibits the uptake of AICAR, so when combined with AICAR, no activation of AMPK is observed because no AICAR is taken by the cells. The same is true for some p38-MAPK inhibitors like SB202190 and SB203580 [148].

C75 (40 μg/ml) (a synthetic inhibitor of fatty acid synthase and an activator of carnitine palmitoyltransferase-1) increases fatty acid oxidation and ATP production leading to the inhibition of AMPK. The C75-mediated inactivation of AMPK at the hypothalamus may explain the food-intake reducing effects of C75 ([149], [150]).

Leptin and α-lipoic acid both are able to decrease hypothalamic AMPK activity, causing profound weight loss in rodents by reducing food intake and enhancing energy expenditure ([112], [115], [116]).

High levels of **glycogen** decrease AMPK activity [151]. High levels of **glucose** also decrease AMPK activity in both β-cells [152] and skeletal muscle [123].

Ethanol also inhibits liver AMPK. This inhibition accounts for the observed activation of hepatic ACC and development of alcoholic fatty acid liver [153].

Insulin antagonizes the activation of AMPK. The effect of insulin (that was blocked by wortmannin, an inhibitor of phosphatidylinositol-3-kinase) occurred only if insulin was added before AMPK activation, and resulted in a decreased phosphorylation of AMPK Thr172, being unrelated to changes in the AMP:ATP ratio [154]. In cardiac cells, inhibition by insulin is dependent on Akt; insulin caused an increased Akt phosphorylation (mediated by PI3-kinase) and a decrease in AMPK phosphorylation at Thr172; in fact, a constitutively active form of Akt displayed decreased phosphorylation of AMPK [155]. It has been recently reported that Akt is able to phosphorylate AMPK-α subunit at Ser485α1/491α2 site. This phosphorylation prevents LKB1 phosphorylation at SerT172, decreasing the activity of AMPK ([49], [50]).

cAMP-PKA can also phosphorylate AMPK-α at Ser485α1/491α2 site, decreasing then the phosphorylation at Thr172 and AMPK activity. cAMP agonists such as forskolin, isobutylmethylxanthine and glucose-dependent insulinotropic peptide (GIP) inhibit AMPK by this mechanism [51]. Is has also been proposed that the Ser485α1/491α2 site could be a site for autophosphorylation, playing a role in limiting AMPK activation in response to energy depletion or other regulators [51].

Resistin, another adipokine, seems to have opposite effects to adiponectin. It inhibits AMPK. Knock-out mice lacking resistin have low blood glucose due to reduced glucose production by the liver, and this is associated with activation of AMPK and reduced expression of enzymes of gluconeogenesis ([112], [156]).

Staurosporine (2.9 nM) [157], **Iodotubercidin** (10 μM) [158] and **9-β-D-arabinofuranoside (ara-A)** (2.5 mM) [158] have been used as inhibitors of AMPK. However they are not specific since they can inhibit additional protein kinases.

8.- PERSPECTIVES

Although a lot of efforts have been made to understand the structure and regulation of the activity of AMPK complex, there are still some areas of missing information. For example, only partial crystal structures of the complex are available and they all lack the kinase catalytic domain and the autoinhibitory region. It would be very interesting to see how the kinase catalytic subunit folds around the other components of the heterotrimeric complex and validate the biophysical results already obtained. These results will allow us to understand to relationship between the kinase catalytic domain and the autoinhibitory region and how the CBS domains of γ subunit play a role in the activation of the kinase subunit. This information will be very important for the design of specific drugs that could either activate or inactivate specific AMPK complexes in targeted tissues.

9.- ACKNOWLEDGEMENTS

I want to thank Dr. L. Yenush and Dr. M.A. Garcia-Gimeno for the critical reading of the manuscript and Dr. Alberto Marina for the help in the structural analysis. This work has been supported by the Research and Technological Development Project LSHM-CT-2004-005272 funded by the European Commission, the Spanish Ministry of

Education and Science grant SAF2005-00852 and grants from the CIBER de Enfermedades Raras, an initiative of the ISCIII.

10.- REFERENCES

[1] Carling, D. (**2004**) *Trends Biochem Sci,* 29, 18-24

[2] Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (**2005**) *Cell Metab,* 1, 15-25

[3] Hardie, D. G. (**2005**) *Curr Opin Cell Biol,* 17, 167-173

[4] Hardie, D. G., and Sakamoto, K. (**2006**) *Physiology (Bethesda),* 21, 48-60

[5] Hardie, D. G. (**2007**) *Nature reviews,* 8, 774-785

[6] Hardie, D. G., Carling, D., and Carlson, M. (**1998**) *Annu Rev Biochem,* 67, 821-855

[7] Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (**2003**) *FEBS Lett,* 546, 113-120

[8] Salt, I. P., Johnson, G., Ashcroft, S. J., and Hardie, D. G. (**1998**) *Biochem J,* 335, 533-539.

[9] Bateman, A. (**1997**) *Trends Biochem Sci,* 22, 12-13.

[10] Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (**2004**) *J Clin Invest,* 113, 274-284

[11] Thornton, C., Snowden, M. A., and Carling, D. (**1998**) *J Biol Chem,* 273, 12443-12450.

[12] Gimeno-Alcaniz, J. V., and Sanz, P. (**2003**) *J Mol Biol,* 333, 201-209

[13] Jorgensen, S. B., Richter, E. A., and Wojtaszewski, J. F. (**2006**) *J Physiol,* 574, 17-31

[14] Dyck, J. R., and Lopaschuk, G. D. (**2006**) *J Physiol,* 574, 95-112

[15] Viollet, B., Foretz, M., Guigas, B., Horman, S., Dentin, R., Bertrand, L., Hue, L., and Andreelli, F. (**2006**) *J Physiol,* 574, 41-53

[16] Daval, M., Foufelle, F., and Ferre, P. (**2006**) *J Physiol,* 574, 55-62

[17] Xue, B., and Kahn, B. B. (**2006**) *J Physiol,* 574, 73-83

[18] Reznick, R. M., and Shulman, G. I. (**2006**) *J Physiol,* 574, 33-39

[19] Motoshima, H., Goldstein, B. J., Igata, M., and Araki, E. (**2006**) *J Physiol,* 574, 63-71

[20] Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., and Neumann, D. (**2006**) *J Biol Chem,* 281, 32207-32216

[21] Jiang, R., and Carlson, M. (**1996**) *Genes Develop.,* 10, 3105-3115

[22] Leech, A., Nath, N., McCartney, R. R., and Schmidt, M. C. (**2003**) *Eukaryot Cell,* 2, 265-273

[23] Pang, T., Xiong, B., Li, J. Y., Qiu, B. Y., Jin, G. Z., Shen, J. K., and Li, J. (**2007**) *J Biol Chem,* 282, 495-506

[24] Scott, J. W., Norman, D. G., Hawley, S. A., Kontogiannis, L., and Hardie, D. G. (**2002**) *J Mol Biol,* 317, 309-323.

[25] Pang, T., Zhang, Z. S., Gu, M., Qiu, B. Y., Yu, L. F., Cao, P. R., Shao, W., Su, M. B., Li, J. Y., Nan, F. J., and Li, J. (**2008**) *J Biol Chem,* in press.

[26] Rudolph, M. J., Amodeo, G. A., Bai, Y., and Tong, L. (**2005**) *Biochem Biophys Res Commun,* 337, 1224-1228

[27] Nayak, V., Zhao, K., Wyce, A., Schwartz, M. F., Lo, W. S., Berger, S. L., and Marmorstein, R. (**2006**) *Structure,* 14, 477-485

[28] Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (**2003**) *Curr Biol,* 13, 861-866

[29] Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., Kemp, B. E., and Stapleton, D. (**2003**) *Curr Biol,* 13, 867-871

[30] Warden, S. M., Richardson, C., O'Donnell, J., Jr., Stapleton, D., Kemp, B. E., and Witters, L. A. (**2001**) *Biochem J,* 354, 275-283.

[31] Mitchelhill, K. I., Michell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A., and Kemp, B. E. (**1997**) *J Biol Chem,* 272, 24475-24479.

[32] Polekhina, G., Gupta, A., van Denderen, B. J., Feil, S. C., Kemp, B. E., Stapleton, D., and Parker, M. W. (**2005**) *Structure,* 13, 1453-1462

[33] Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G., and Carling, D. (**2000**) *Biochem J,* 346, 659-669.

[34] Viana, R., Towler, M. C., Pan, D. A., Carling, D., Viollet, B., Hardie, D. G., and Sanz, P. (**2007**) *J Biol Chem,* 282, 16117-16125

[35] Day, P., Sharff, A., Parra, L., Cleasby, A., Williams, M., Horer, S., Nar, H., Redemann, N., Tickle, I., and Yon, J. (**2007**) *Acta crystallographica,* 63, 587-596

[36] Meyer, S., and Dutzler, R. (**2006**) *Structure,* 14, 299-307

[37] Meyer, S., Savaresi, S., Forster, I. C., and Dutzler, R. (**2007**) *Nat Struct Mol Biol,* 14, 60-67

[38] Rudolph, M. J., Amodeo, G. A., Iram, S. H., Hong, S. P., Pirino, G., Carlson, M., and Tong, L. (**2007**) *Structure,* 15, 65-74

[39] Dyck, J. R., Gao, G., Widmer, J., Stapleton, D., Fernandez, C. S., Kemp, B. E., and Witters, L. A. (**1996**) *J Biol Chem,* 271, 17798-17803.

[40] Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (**1998**) *J Biol Chem,* 273, 35347-35354.

[41] Woods, A., Cheung, P. C., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., and Carling, D. (**1996**) *J Biol Chem,* 271, 10282-10290.

[42] Iseli, T. J., Walter, M., van Denderen, B. J., Katsis, F., Witters, L. A., Kemp, B. E., Michell, B. J., and Stapleton, D. (**2005**) *J Biol Chem,* 280, 13395-13400

[43] Elbing, K., Rubenstein, E. M., McCartney, R. R., and Schmidt, M. C. (**2006**) *J Biol Chem,* 281, 26170-26180

[44] Wong, K. A., and Lodish, H. F. (**2006**) *J Biol Chem,* 281, 36434-36442

[45] Mu, J., Brozinick, J. T., Jr., Valladares, O., Bucan, M., and Birnbaum, M. J. (**2001**) *Mol Cell,* 7, 1085-1094.

[46] Iseli, T. J., Oakhill, J. S., Bailey, M. F., Wee, S., Walter, M., van Denderen, B. J., Castelli, L. A., Katsis, F., Witters, L. A., Stapleton, D., Macaulay, S. L., Michell, B. J., and Kemp, B. E. (**2008**) *J Biol Chem,* 283, 4799-4807

[47] Gissot, L., Polge, C., Bouly, J. P., Lemaitre, T., Kreis, M., and Thomas, M. (**2004**) *Plant Mol Biol,* 56, 747-759

[48] Woods, A., Vertommen, D., Neumann, D., Turk, R., Bayliss, J., Schlattner, U., Wallimann, T., Carling, D., and Rider, M. H. (**2003**) *J Biol Chem,* 278, 28434-28442

[49] Horman, S., Vertommen, D., Heath, R., Neumann, D., Mouton, V., Woods, A., Schlattner, U., Wallimann, T., Carling, D., Hue, L., and Rider, M. H. (**2006**) *J Biol Chem,* 281, 5335-5340

[50] Soltys, C. L., Kovacic, S., and Dyck, J. R. (**2006**) *Am J Physiol Heart Circ Physiol,* 290, H2472-2479

[51] Hurley, R. L., Barre, L. K., Wood, S. D., Anderson, K. A., Kemp, B. E., Means, A. R., and Witters, L. A. (**2006**) *J Biol Chem,* 281, 36662-36672

[52] Townley, R., and Shapiro, L. (**2007**) *Science,* 315, 1726-1729

[53] Xiao, B., Heath, R., Saiu, P., Leiper, F. C., Leone, P., Jing, C., Walker, P. A., Haire, L., Eccleston, J. F., Davis, C. T., Martin, S. R., Carling, D., and Gamblin, S. J. (**2007**) *Nature,* 449, 496-500

[54] Amodeo, G. A., Rudolph, M. J., and Tong, L. (**2007**) *Nature,* 449, 492-495

[55] Jin, X., Townley, R., and Shapiro, L. (**2007**) *Structure,* 15, 1285-1295

[56] Arad, M., Seidman, C. E., and Seidman, J. G. (**2007**) *Circ Res,* 100, 474-488

[57] Riek, U., Scholz, R., Konarev, P., Rufer, A., Suter, M., Nazabal, A., Ringler, P., Chami, M., Muller, S. A., Neumann, D., Forstner, M., Hennig, M., Zenobi, R., Engel, A., Svergun, D., Schlattner, U., and Wallimann, T. (**2008**) *J Biol Chem,* in press

[58] Daniel, T. D., and Carling, D. (**2002**) *Biochem J,* 365, 629-638

[59] Wilson, W. A., Hawley, S. A., and Hardie, D. G. (**1996**) *Curr Biol,* 6, 1426-1434.

[60] Halford, N. G., and Hardie, D. G. (**1998**) *Plant Mol Biol,* 37, 735-748.

[61] Hardie, D. G., Salt, I. P., Hawley, S. A., and Davies, S. P. (**1999**) *Biochem J,* 338, 717-722.

[62] Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A., and Carling, D. (**2007**) *Biochem J,* 403, 139-148

[63] Sugden, C., Crawford, R. M., Halford, N. G., and Hardie, D. G. (**1999**) *Plant J,* 19, 433-439.

[64] Hardie, D. G., and Hawley, S. A. (**2001**) *Bioessays,* 23, 1112-1119.

[65] Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (**2000**) *Biochem J,* 345 Pt 3, 437-443.

[66] McCartney, R. R., and Schmidt, M. C. (**2001**) *J Biol Chem,* 276, 36460-36466.

[67] Nath, N., McCartney, R. R., and Schmidt, M. C. (**2003**) *Mol Cell Biol,* 23, 3909-3917

[68] Hong, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (**2003**) *Proc Natl Acad Sci U S A,* 100, 8839-8843

[69] Sutherland, C. M., Hawley, S. A., McCartney, R. R., Leech, A., Stark, M. J., Schmidt, M. C., and Hardie, D. G. (**2003**) *Curr Biol,* 13, 1299-1305

[70] Hedbacker, K., Hong, S. P., and Carlson, M. (**2004**) *Mol Cell Biol,* 24, 8255-8263

[71] McCartney, R. R., Rubenstein, E. M., and Schmidt, M. C. (**2005**) *Curr Genet,* 47, 335-344

[72] Elbing, K., McCartney, R. R., and Schmidt, M. C. (**2006**) *Biochem J,* 393, 797-805

[73] Hunter, T., and Plowman, G. D. (**1997**) *Trends Biochem Sci,* 22, 18-22

[74] Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (**2003**) *J Biol,* 2, 28

[75] Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (**2003**) *Curr Biol,* 13, 2004-2008

[76] Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (**2005**) *Cell Metab,* 2, 21-33

[77] Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (**2005**) *J Biol Chem,* 280, 29060-29066

[78] Hong, S. P., Momcilovic, M., and Carlson, M. (**2005**) *J Biol Chem,* 280, 21804-21809

[79] Momcilovic, M., Hong, S. P., and Carlson, M. (**2006**) *J Biol Chem,* 281, 25336-25343

[80] Alessi, D. R., Sakamoto, K., and Bayascas, J. R. (**2006**) *Annu Rev Biochem,* 75, 137-163

[81] Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R., and Clevers, H. C. (**2003**) *Embo J,* 22, 3062-3072

[82] Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C., and Alessi, D. R. (**2003**) *Embo J,* 22, 5102-5114

[83] Milburn, C. C., Boudeau, J., Deak, M., Alessi, D. R., and van Aalten, D. M. (**2004**) *Nat Struct Mol Biol,* 11, 193-200

[84] Boudeau, J., Scott, J. W., Resta, N., Deak, M., Kieloch, A., Komander, D., Hardie, D. G., Prescott, A. R., van Aalten, D. M., and Alessi, D. R. (**2004**) *J Cell Sci,* 117, 6365-6375

[85] Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (**2004**) *Proc Natl Acad Sci U S A,* 101, 3329-3335

[86] Baas, A. F., Smit, L., and Clevers, H. (**2004**) *Trends Cell Biol,* 14, 312-319

[87] Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A., and Alessi, D. R. (**2005**) *Embo J,* 24, 1810-1820

[88] Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., and Cantley, L. C. (**2005**) *Science,* 310, 1642-1646

[89] Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., and Moller, D. E. (**2001**) *J Clin Invest,* 108, 1167-1174.

[90] Altarejos, J. Y., Taniguchi, M., Clanachan, A. S., and Lopaschuk, G. D. (**2005**) *J Biol Chem,* 280, 183-190

[91] Suzuki, A., Kusakai, G., Kishimoto, A., Shimojo, Y., Ogura, T., Lavin, M. F., and Esumi, H. (**2004**) *Biochem Biophys Res Commun,* 324, 986-992

[92] Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (**2005**) *Cell Metab,* 2, 9-19

[93] Witters, L. A., Kemp, B. E., and Means, A. R. (**2006**) *Trends Biochem Sci,* 31, 13-16

[94] Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (**1995**) *Eur J Biochem,* 229, 558-565.

[95] Rutter, G. A., Da Silva Xavier, G., and Leclerc, I. (**2003**) *Biochem J,* 375, 1-16

[96] Shang, J., and Lehrman, M. A. (**2004**) *J Biol Chem,* 279, 12076-12080

[97] Gadalla, A. E., Pearson, T., Currie, A. J., Dale, N., Hawley, S. A., Sheehan, M., Hirst, W., Michel, A. D., Randall, A., Hardie, D. G., and Frenguelli, B. G. (**2004**) *J Neurochem,* 88, 1272-1282

[98] Guigas, B., Bertrand, L., Taleux, N., Foretz, M., Wiernsperger, N., Vertommen, D., Andreelli, F., Viollet, B., and Hue, L. (**2006**) *Diabetes,* 55, 865-874

[99] Guigas, B., Taleux, N., Foretz, M., Detaille, D., Andreelli, F., Viollet, B., and Hue, L. (**2007**) *Biochem J,* 404, 499-507

[100] Jacobs, R. L., Lingrell, S., Dyck, J. R., and Vance, D. E. (**2007**) *J Biol Chem,* 282, 4516-4523

[101] Viana, R., Aguado, C., Esteban, I., Moreno, D., Viollet, B., Knecht, E., and Sanz, P. (**2008**) *Biochem Biophys Res Commun,* 369, 964-968

[102] Kuo, C. L., Ho, F. M., Chang, M. Y., Prakash, E., and Lin, W. W. (**2008**) *Journal of cellular biochemistry,* 103, 931-940

[103] Fryer, L. G., Parbu-Patel, A., and Carling, D. (**2002**) *J Biol Chem,* 277, 25226-25232

[104] Almeida, A., Cidad, P., Delgado-Esteban, M., Fernandez, E., Garcia-Nogales, P., and Bolanos, J. P. (**2005**) *J Neurosci Res,* 79, 166-171

[105] Choi, S. L., Kim, S. J., Lee, K. T., Kim, J., Mu, J., Birnbaum, M. J., Soo Kim, S., and Ha, J. (**2001**) *Biochem Biophys Res Commun,* 287, 92-97.

[106] Dokladda, K., Green, K. A., Pan, D. A., and Hardie, D. G. (**2005**) *FEBS Lett,* 579, 236-240

[107] Brunmair, B., Staniek, K., Gras, F., Scharf, N., Althaym, A., Clara, R., Roden, M., Gnaiger, E., Nohl, H., Waldhausl, W., and Furnsinn, C. (**2004**) *Diabetes,* 53, 1052-1059

[108] Saha, A. K., Avilucea, P. R., Ye, J. M., Assifi, M. M., Kraegen, E. W., and Ruderman, N. B. (**2004**) *Biochem Biophys Res Commun,* 314, 580-585

[109] Pilon, G., Dallaire, P., and Marette, A. (**2004**) *J Biol Chem,* 279, 20767-20774

[110] Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (**2002**) *Nature,* 415, 339-343.

[111] Yu, X., McCorkle, S., Wang, M., Lee, Y., Li, J., Saha, A. K., Unger, R. H., and Ruderman, N. B. (**2004**) *Diabetologia,* 47, 2012-2021

[112] Kola, B., Boscaro, M., Rutter, G. A., Grossman, A. B., and Korbonits, M. (**2006**) *Trends Endocrinol Metab,* 17, 205-215

[113] Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (**2002**) *Nat Med,* 8, 1288-1295.

[114] Shibata, R., Ouchi, N., Ito, M., Kihara, S., Shiojima, I., Pimentel, D. R., Kumada, M., Sato, K., Schiekofer, S., Ohashi, K., Funahashi, T., Colucci, W. S., and Walsh, K. (**2004**) *Nat Med,* 10, 1384-1389

[115] Lee, W. J., Song, K. H., Koh, E. H., Won, J. C., Kim, H. S., Park, H. S., Kim, M. S., Kim, S. W., Lee, K. U., and Park, J. Y. (**2005**) *Biochem Biophys Res Commun,* 332, 885-891

[116] Kim, M. S., Park, J. Y., Namkoong, C., Jang, P. G., Ryu, J. W., Song, H. S., Yun, J. Y., Namgoong, I. S., Ha, J., Park, I. S., Lee, I. K., Viollet, B., Youn, J. H., Lee, H. K., and Lee, K. U. (**2004**) *Nat Med,* 10, 727-733

[117] Zou, M. H., Hou, X. Y., Shi, C. M., Nagata, D., Walsh, K., and Cohen, R. A. (**2002**) *J Biol Chem,* 277, 32552-32557.

[118] Zou, M. H., Hou, X. Y., Shi, C. M., Kirkpatick, S., Liu, F., Goldman, M. H., and Cohen, R. A. (**2003**) *J Biol Chem,* 278, 34003-34010

[119] Witters, L. A. (**2001**) *J Clin Invest,* 108, 1105-1107.

[120] Zou, M. H., Kirkpatrick, S. S., Davis, B. J., Nelson, J. S., Wiles, W. G. t., Schlattner, U., Neumann, D., Brownlee, M., Freeman, M. B., and Goldman, M. H. (**2004**) *J Biol Chem,* 279, 43940-43951

[121] Barnes, K., Ingram, J. C., Porras, O. H., Barros, L. F., Hudson, E. R., Fryer, L. G., Foufelle, F., Carling, D., Hardie, D. G., and Baldwin, S. A. (**2002**) *J Cell Sci,* 115, 2433-2442.

[122] Schulz, E., Anter, E., Zou, M. H., and Keaney, J. F., Jr. (**2005**) *Circulation,* 111, 3473-3480

[123] Itani, S. I., Saha, A. K., Kurowski, T. G., Coffin, H. R., Tornheim, K., and Ruderman, N. B. (**2003**) *Diabetes,* 52, 1635-1640

[124] Fediuc, S., Gaidhu, M. P., and Ceddia, R. B. (**2006**) *J Lipid Res,* 47, 412-420

[125] Clark, H., Carling, D., and Saggerson, D. (**2004**) *Eur J Biochem,* 271, 2215-2224

[126] da Silva, C. G., Jarzyna, R., Specht, A., and Kaczmarek, E. (**2006**) *Circ Res,* 98, e39-47

[127] Aymerich, I., Foufelle, F., Ferre, P., Casado, F. J., and Pastor-Anglada, M. (**2006**) *J Cell Sci,* 119, 1612-1621

[128] Kishi, K., Yuasa, T., Minami, A., Yamada, M., Hagi, A., Hayashi, H., Kemp, B. E., Witters, L. A., and Ebina, Y. (**2000**) *Biochem Biophys Res Commun,* 276, 16-22.

[129] Hutchinson, D. S., and Bengtsson, T. (**2006**) *Diabetes,* 55, 682-690

[130] Yin, W., Mu, J., and Birnbaum, M. J. (**2003**) *J Biol Chem,* 278, 43074-43080

[131] Hutchinson, D. S., Chernogubova, E., Dallner, O. S., Cannon, B., and Bengtsson, T. (**2005**) *Diabetologia,* 48, 2386-2395

[132] Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R., Zhao, G., Marsh, K., Kym, P., Jung, P., Camp, H. S., and Frevert, E. (**2006**) *Cell Metab,* 3, 403-416

[133] Sanders, M. J., Ali, Z. S., Hegarty, B. D., Heath, R., Snowden, M. A., and Carling, D. (**2007**) *J Biol Chem,* 282, 32539-32548

[134] Goransson, O., McBride, A., Hawley, S. A., Ross, F. A., Shpiro, N., Foretz, M., Viollet, B., Hardie, D. G., and Sakamoto, K. (**2007**) *J Biol Chem,* 282, 32549-32560

[135] Lee, Y. S., Kim, W. S., Kim, K. H., Yoon, M. J., Cho, H. J., Shen, Y., Ye, J. M., Lee, C. H., Oh, W. K., Kim, C. T., Hohnen-Behrens, C., Gosby, A., Kraegen, E. W., James, D. E., and Kim, J. B. (**2006**) *Diabetes,* 55, 2256-2264

[136] Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Le Couteur, D., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., de Cabo, R., and Sinclair, D. A. (**2006**) *Nature,* 444, 337-342

[137] Collins, Q. F., Liu, H. Y., Pi, J., Liu, Z., Quon, M. J., and Cao, W. (**2007**) *J Biol Chem,* 282, 30143-30149

[138] Lin, C. L., Huang, H. C., and Lin, J. K. (**2007**) *J Lipid Res,* 48, 2334-2343

[139] Lee, E. S., Uhm, K. O., Lee, Y. M., Han, M., Lee, M., Park, J. M., Suh, P. G., Park, S. H., and Kim, H. S. (**2007**) *Biochem Biophys Res Commun,* 361, 854-858

[140] Tan, M. J., Ye, J. M., Turner, N., Hohnen-Behrens, C., Ke, C. Q., Tang, C. P., Chen, T., Weiss, H. C., Gesing, E. R., Rowland, A., James, D. E., and Ye, Y. (**2008**) *Chem Biol,* 15, 263-273

[141] Ludin, K., Jiang, R., and Carlson, M. (**1998**) *Proc. Natl. Acad. Sci. USA,* 95, 6245-6250

[142] Sanz, P., Alms, G. R., Haystead, T. A., and Carlson, M. (**2000**) *Mol Cell Biol,* 20, 1321-1328

[143] Davies, S. P., Helps, N. R., Cohen, P. T., and Hardie, D. G. (**1995**) *FEBS Lett,* 377, 421-425.

[144] Samari, H. R., Moller, M. T., Holden, L., Asmyhr, T., and Seglen, P. O. (**2005**) *Biochem J,* 386, 237-244

[145] Wu, Y., Song, P., Xu, J., Zhang, M., and Zou, M. H. (**2007**) *J Biol Chem,* 282, 9777-9788

[146] Woods, A., Azzout-Marniche, D., Foretz, M., Stein, S. C., Lemarchand, P., Ferre, P., Foufelle, F., and Carling, D. (**2000**) *Mol Cell Biol,* 20, 6704-6711.

[147] Emerling, B. M., Viollet, B., Tormos, K. V., and Chandel, N. S. (**2007**) *FEBS Lett,* 581, 5727-5731

[148] Fryer, L. G., Parbu-Patel, A., and Carling, D. (**2002**) *FEBS Lett,* 531, 189-192

[149] Landree, L. E., Hanlon, A. L., Strong, D. W., Rumbaugh, G., Miller, I. M., Thupari, J. N., Connolly, E. C., Huganir, R. L., Richardson, C., Witters, L. A., Kuhajda, F. P., and Ronnett, G. V. (**2004**) *J Biol Chem,* 279, 3817-3827

[150] Kim, E. K., Miller, I., Aja, S., Landree, L. E., Pinn, M., McFadden, J., Kuhajda, F. P., Moran, T. H., and Ronnett, G. V. (**2004**) *J Biol Chem,* 279, 19970-19976

[151] Winder, W. W. (**2001**) *J Appl Physiol,* 91, 1017-1028.

[152] da Silva Xavier, G., Leclerc, I., Varadi, A., Tsuboi, T., Moule, S. K., and Rutter, G. A. (**2003**) *Biochem J,* 371, 761-774

[153] You, M., Matsumoto, M., Pacold, C. M., Cho, W. K., and Crabb, D. W. (**2004**) *Gastroenterology,* 127, 1798-1808

[154] Beauloye, C., Marsin, A. S., Bertrand, L., Krause, U., Hardie, D. G., Vanoverschelde, J. L., and Hue, L. (**2001**) *FEBS Lett,* 505, 348-352.

[155] Kovacic, S., Soltys, C. L., Barr, A. J., Shiojima, I., Walsh, K., and Dyck, J. R. (**2003**) *J Biol Chem,* 278, 39422-39427

[156] Banerjee, R. R., Rangwala, S. M., Shapiro, J. S., Rich, A. S., Rhoades, B., Qi, Y., Wang, J., Rajala, M. W., Pocai, A., Scherer, P. E., Steppan, C. M., Ahima, R. S., Obici, S., Rossetti, L., and Lazar, M. A. (**2004**) *Science,* 303, 1195-1198

[157] Anderson, S. N., Cool, B. L., Kifle, L., Chiou, W., Egan, D. A., Barrett, L. W., Richardson, P. L., Frevert, E. U., Warrior, U., Kofron, J. L., and Burns, D. J. (**2004**) *J Biomol Screen,* 9, 112-121

[158] Musi, N., Hayashi, T., Fujii, N., Hirshman, M. F., Witters, L. A., and Goodyear, L. J. (**2001**) *Am J Physiol Endocrinol Metab,* 280, E677-684.

Table I: Characteristics of different structures of AMPK heterotrimeric complexes.

	Townley et al [52]	**Xiao et al** [53]	**Amodeo et al** [54]
Organism	*Schizosaccharomyces pombe*	Rattus novergicus	*Saccharomyces cerevisiae*
PDB number	2OOX	2V8Q	2QLV
Components (residues in brackets)	α (440-576) β (205-298) γ(1-334; full length)	α1(396-548) β2 (187-272) γ1(1-331; full length)	Snf1(398-633) Sip2(154-415) Snf4 (1-322; full length)
Overall structure	Dimer of heterotrimers	Monomer of heterotrimer	Dimer of heterotrimers
Structure of monomer	Triangular	Triangular. α and β subunits twisted 12º respect to *S. pombe* structure	Triangular. α and β subunits twisted 12º respect to *S. pombe* structure
Number of AMP bound	1 (AMP3 site)	3 (AMP1, AMP2 and AMP3 sites)	1 (AMP3 site)
Additional features			Snf1 regulatory domain bound to CBS4 region of Snf4; Sip2 GBD bound to CBS2 region of Snf4

Table II: Methods for activating AMPK

AMPK activation affecting AMP/ATP ratio	**Reference**	**AMPK activation without altering AMP/ATP ratio**	**Reference**
AICAR	[94]	Peroxynitrite	[117]
Dinitrophenol	[103]	Metformin	[120]
H_2O_2	[105]	Osmotic stress	[58]
Inhibitors of MAP kinase cascade	[106]	Estradiol	[122]
Thiazolidinediones	[103]	Low glucose	[123]
Leptin (in muscle)	[110]	Long chain saturated and monosaturated fatty acids	[124]
Adiponectin	[113]	Agonists of nucleotide receptors	[126]
α-Lipoic acid (in muscle)	[115]	Agonists of α1-adrenoreceptors	[129]
		A769662	[132]

Table III: Methods for inactivating AMPK

AMPK inactivation	**Reference**
Dominant negative forms of AMPK-α	[40], [45]
Creatine phosphate and ATP	[60], [64]
Compound C	[89], [148]
C75 (hypothalamus)	[149], [150]
Leptin (hypothalamus)	[112]
α-Lipoic acid (hypothalamus)	[116], [115]
Glycogen	[151]
High glucose	[152], [123]
Ethanol	[153]
Staurosporine	[157]
Iodotubercidin	[158]
9-b-D-arabinofuranoside	[158]
Insulin (heart)	[154], [155]
Agonists of cAMP-PKA pathway	[51]
Resistin	[156]

FIGURE LEGENDS

Fig. 1: Diagram of mammalian AMPK and yeast SNF1 subunits. KD, kinase domain; RD, regulatory domain; AID, auto-inhibitory domain; GBD, glycogen bind domain; ASC, association with Snf1 complex domain; Myr, myristoylation; CBS, cystathionine-β-synthase domain.

Fig. 2: Crystal structure models of different kinase domains of α subunits. The crystal structure of the kinase domains of Snf1 (PDB, 2EUE), (PDB, 2FH9) and AMPK-α2 (PDB, 2H6D) were modeled using the PyMol programme (DeLano Scientific). The small N-terminal and the large C-terminal domains are indicated. In the case of the 2FH9 model, the second protomer in colored in yellow. The position of K45 and D157 residues in AMPK-α2 subunit is also indicated.

Fig. 3: A) The crystal structure model of GBD domain of AMPK-β1 subunit (PDB, 1Z0M) was modeled using the PyMol programme (DeLano Scientific). Residues involved in carbohydrate binding (K126, W133, L146 and N150) and the β-cyclodextrin ring (yellow) are indicated. **B)** Crystal structure model of a CBS domain pair from human AMPK-γ1 (residues 182-325, comprising CBS3 plus CBS4) in complex with AMP (PDB, 2UV4). The conserved βαββα structures of CBS3 (yellow) and CBS4 (purple), the sheet of the three β-strands in each CBS domain and the AMP molecule is indicated.

Fig. 4: A) Crystal structure model of the heterotrimeric core of *Saccharomyces cerevisiae* SNF1 complex (PDB, 2QLV) was modeled using the PyMol programme (DeLano Scientific). α subunit (Snf1) is colored in purple, β subunit (Sip2) is colored in green and γ subunit (Snf4) is colored in blue (CBS1+2) and yellow (CBS3+4). The position of the three-stranded β-sheets from β and γ subunits, the GBD domain of β subunit, the RD domain of the α subunit and the corresponding CBS domains is indicated. B) Crystal structure model of the mammalian AMPK-γ1 subunit (PDB, 2V8Q) in complex with AMP was modeled using the PyMol programme (DeLano Scientific). The conserved βαββα structures of CBS1 (green), CBS2 (yellow), CBS3 (purple) and CBS4 (salmon) were colored on the blue backbone. The position of three AMP molecules is indicated.

Fig. 5: Activity of the SNF1 and AMPK complexes is regulated by phosphorylation and γ-subunit dependent rearrangement. A) Snf1 complex; B) AMPK complex. See text for details.

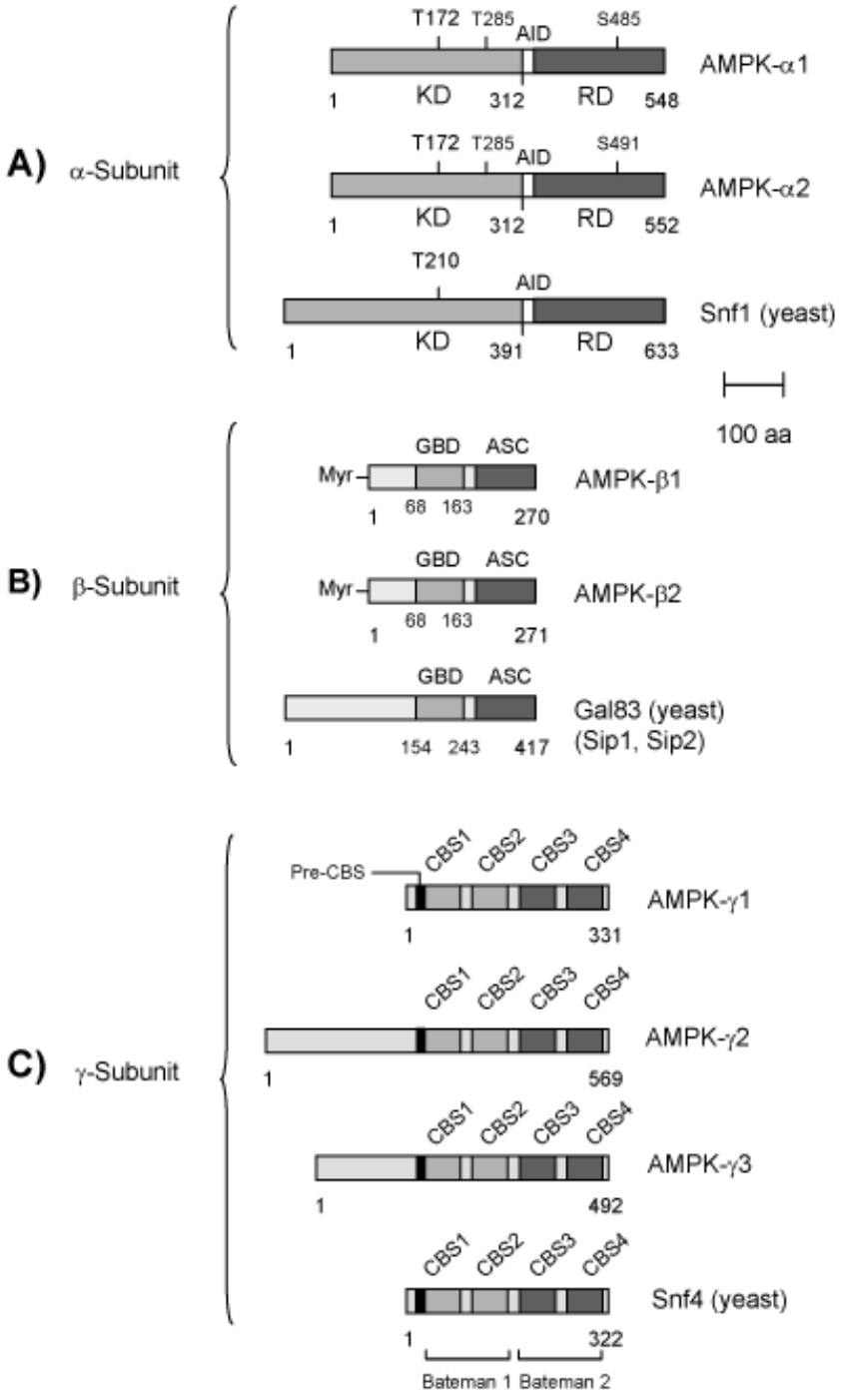

Fig. 1: Sanz (2008).

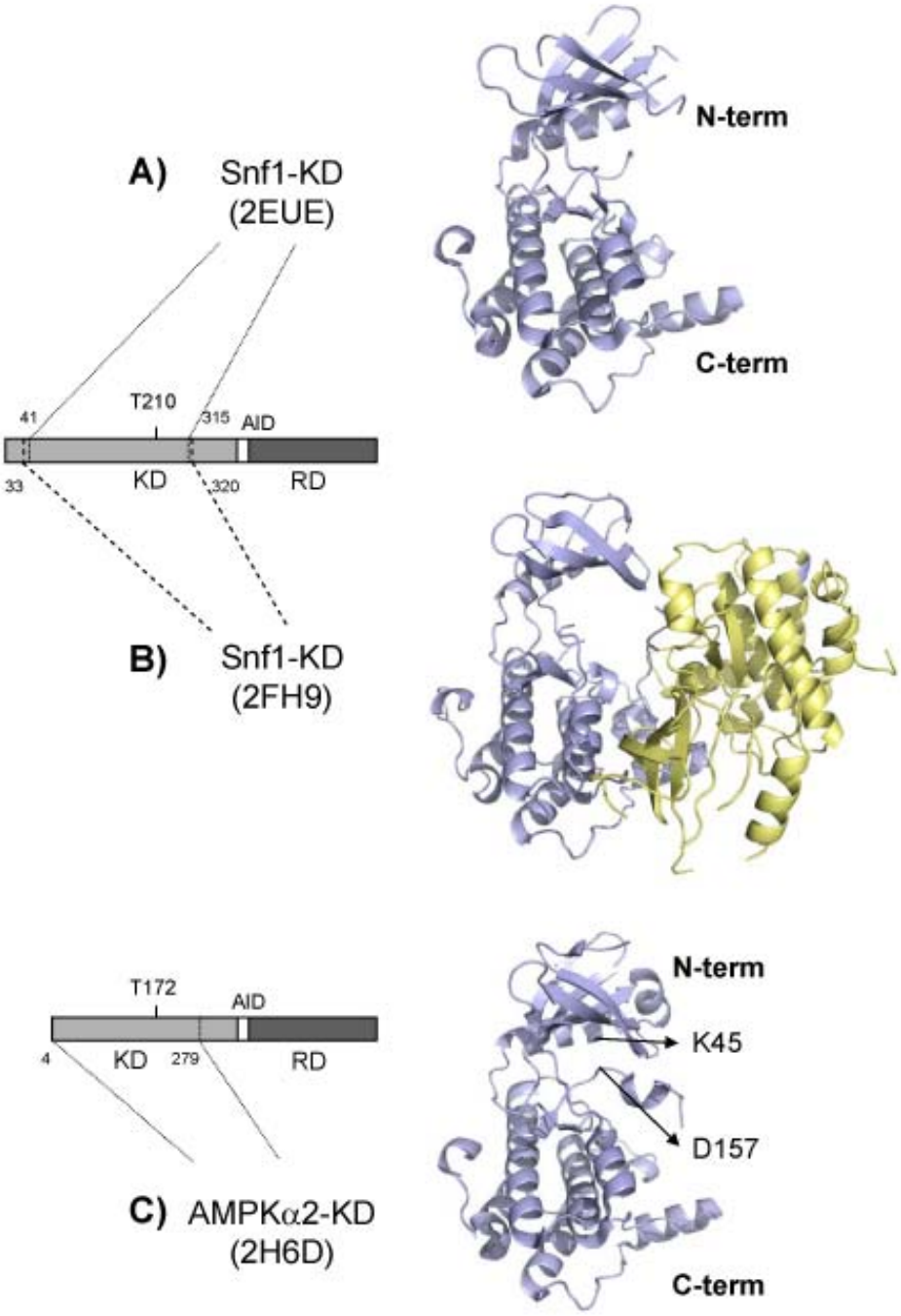

Fig. 2: Sanz (2008)

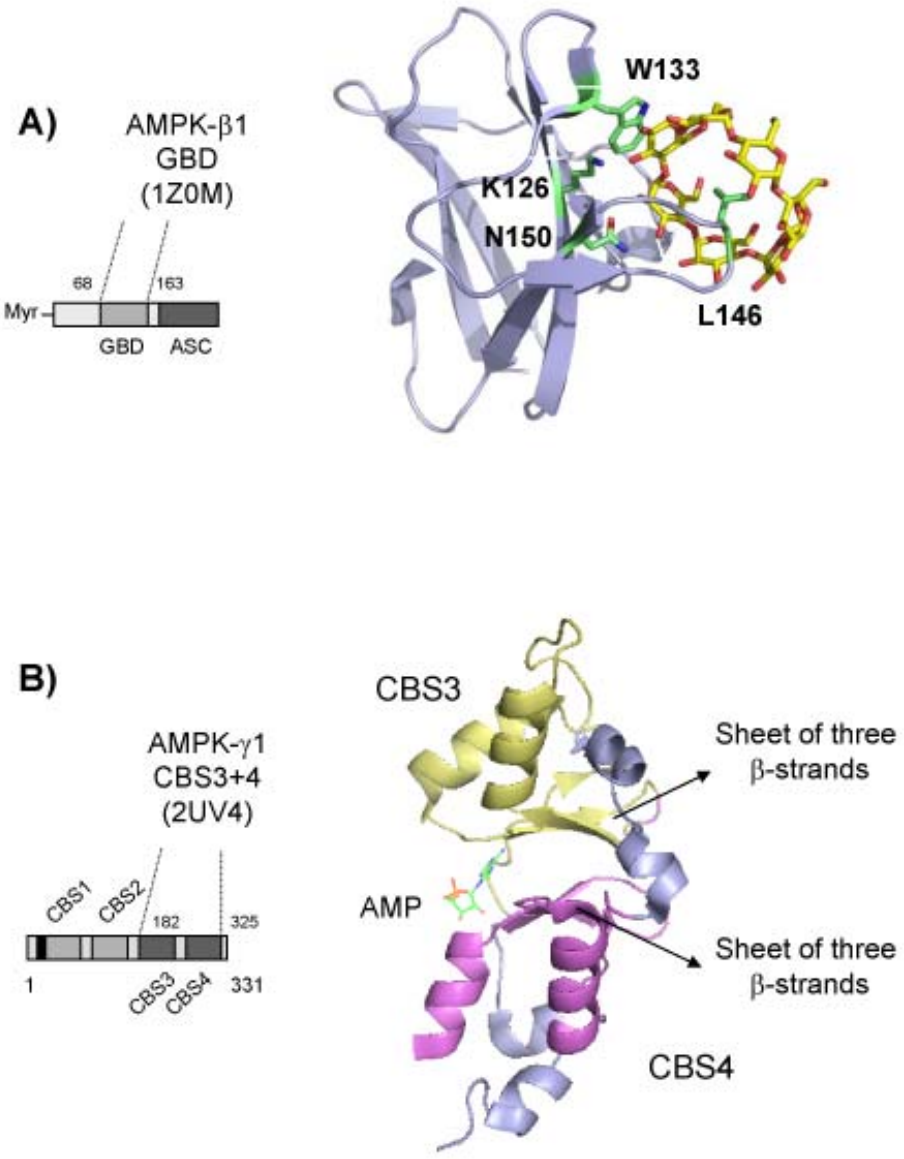

Fig. 3: Sanz (2008)

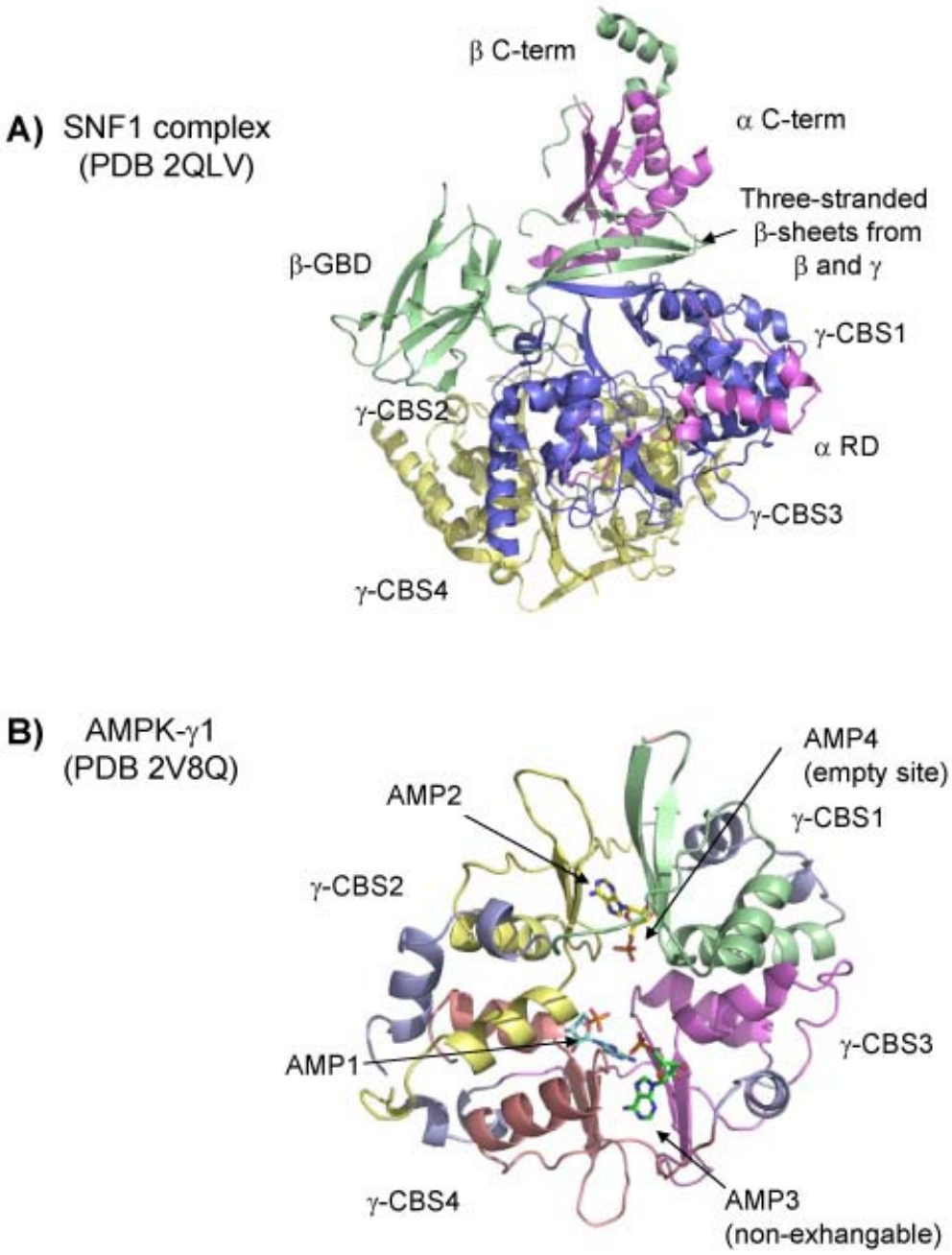

Fig. 4: Sanz (2008)

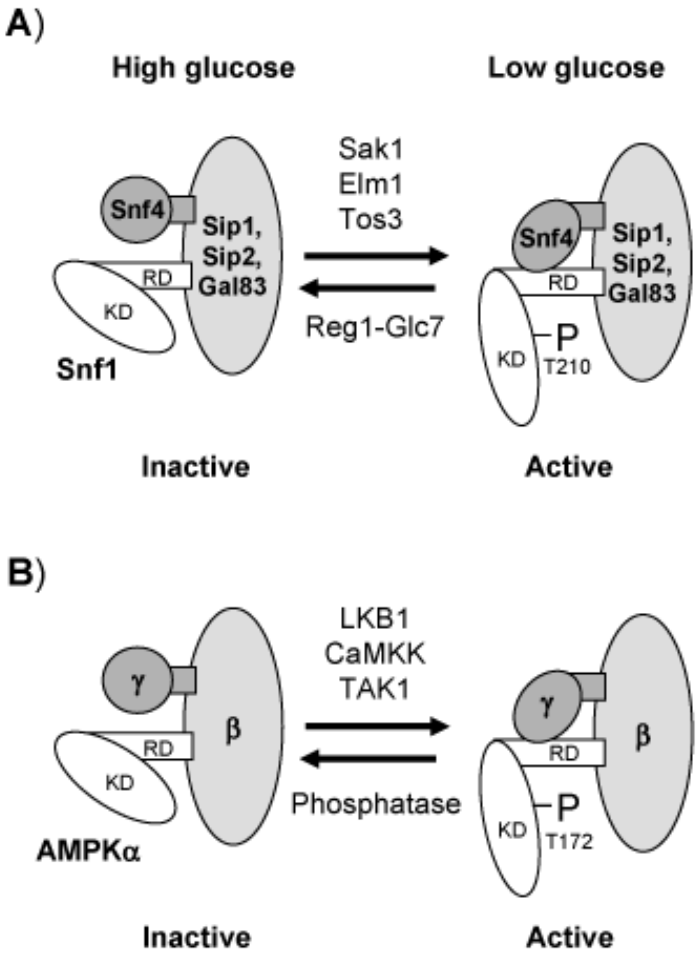

Fig. 5: Sanz (2008).